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DEVELOPMENT OF A GENETICALLY-ENGINEERED VENEZUELAN EQUINE ENCEPHALITIS VIRUS VACCINE

ANNUAL REPORT

DENNIS W. TRENT

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ABSTRACT

Infection of equines with epizootic Venezuelan equine encephalitis (VEE) virus frequently results in fever, leukopenia, meningoencephalitis, and death. Histopathologic changes in the hemolymphatic system include wide spread lymphoid necrosis and depletion of hematopoietic cells in the bone marrow. Changes in the CNS correlated with clinical encephalitis and include diffuse meningoencephalitis with perivascular infiltration of leukocytes, and necrosis. We have evaluated the efficacy of a recombinant vaccinia/VEE virus vaccine (TC-5A) to protect horses against challenge with equine virulent VEE virus. Horses immunized with the vaccinia/VEE virus recombinant virus developed low titer ELISA and neutralizing antibodies after the first immunization. These animals were reimmunized 92 days after the first immunization with recombinant virus to boost the immune response prior to virus challenge. Serum antibody titers seven days following reimmunization were high, indicating antigenic priming had been accomplished by the initial recombinant virus immunization. Horses immunized with the vaccinia/VEE recombinant, wild-type vaccinia virus, and TC-83 vaccine were all challenged with 10^4 PFu of equine virulent VEE virus. Following challenge animals immunized with wild-type vaccinia virus developed a leukopenia, became viremic, febrile, depressed and were euthanized on the sixth day after challenge. Horses immunized with either TC-83 or vaccinia/VEE recombinant viruses did not become viremic or develop clinical or hematologic signs of VEE following challenge. In both groups of immune animals, there was no significant increase in VEE antibody titers following virus challenge, indicating that immunization had fully protected the horses from neuroinvasion.

A full length nucleotide-sequence-perfect cDNA clone of VEE/TC-83 virus has been constructed from fully sequenced subgenomic clones. The full length clone (pVE/Ic-92) is bounded at the 5'-prime end by a unique XbaI site and RNA polymerase T7 promoter. At the 3'-end of the clone there is a poly A tail (n=25), and a unique MluI site which facilitates opening of the plasmid for transcription of the full length virus genome. RNA transcripts synthesized from the cDNA clone in vitro with T7 polymerase are infectious. Unique VEE virus recombinants have been engineered to examine the effect that amino acid differences between the virulent parent virus Trinidad donkey (TRD) and the attenuated TC-83 have on virulence. The Leu to Ile change at amino acid 161 in E1 did not increase the plaque size of the recombinant TC-83 virus in Vero cells. Changes in the E2 glycoprotein at amino acid positions 7, 85, and 120 did not increase the plaque size of the recombinant virus, however, changes at positions 192 and 296 did increase plaque size in Vero cells. The significance of these changes on their virulence in Swiss mice is now being evaluated.

FOREWORD

In conducting research using animals, the investigator adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council (DHHS, PHS, NIH Publication No. 86-23, Revised 1985).

The investigator has abided by the National Institutes of Health Guidelines for Research involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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I. The murine T-helper cell immune response to VEE virus.

Studies of alphavirus cell-mediated immunity have defined the genetic restriction limits for cytotoxic T lymphocytes (CTLs) within the murine major histocompatibility complex (HMC). Mullbacher and Blanden, (1978) recognized the cross-reactive nature of the alphavirus CTL (Mullbacher et al., 1979; Wolcott et al., 1982) and found that protection of mice from virulent virus after adoptive transfer of immune splenocytes is T-cell dependent (Rabinowitz and Adler, 1973; Peck et al., 1975). Indirect evidence for the importance of T-helper (Th) cells in the immune response to alphaviruses was reported by Burns et al. (1975). In this study Sindbis (SIN) virus elicited only a transient T cell-independent IgM response in nude mice; however, only T cell-competent, normal littermates produced IgG and mounted a secondary immune response.

Lymphocyte transformation assays with SIN and VEE virus indicate that T-cells are necessary to induce antigen-specific proliferation (Adler and Rabinowitz, 1973; Griffin and Johnson, 1973; Marker and Ascher, 1976). A virus-specific proliferative response was noted in limited studies with Getah, Ross River, and SIN viruses (Aaskov et al., 1983). We have investigated the antigen-elicited T-cell proliferation by virus-primed T lymphocytes incubated in vitro in the presence of syngeneic stimulator cells previously exposed to virus. Our results indicate that after inoculation of mice with VEE virus, T-cells of the Th

cell phenotype which secrete interleukin-2 (IL-2) are generated against the dominant epitopes that represent virus-specific amino acid sequences and provide help to virus-specific B cell epitopes.

Antigen processing cells (APC) were infected with various concentrations of VEE TC-83 virus to determine the optimal amount of antigen required to induce maximum virus-specific lymphoproliferation. Maximum stimulation of VEE TC-83 virus primed T-cells occurred between 5 and 50 micrograms of virus for ten million splenocytes. Nylon wool(NW) enriched T cells from virus-primed mice proliferated in the presence of phytohemagglutinin to levels comparable to optimal induction levels by purified virus. Virus stimulated the proliferation of T cells in the absence of irradiated APC, at reduced levels indicating that NW chromatography did not remove important APC.

To determine the phenotypes of T cells involved in proliferation, NW enriched T cells from VEE TC-83 or SIN virus-primed mice were treated with T cell marker antisera plus complement (Table 1). Only complement control or cells treated with anti-Lyt-2.1 antibodies proliferated in response to VEE or SIN virus simulation. Similar results were obtained measuring the proliferative response of cells to VEE virus after a culture period of 5 days were treated with antisera, pulsed for 4 hours, and harvested. Both depletion procedures indicated the cell population involved in proliferation was of the Th cell phenotype THy-1(+), Lyt 1(+), 2(-), and L3T4(+). Flow immunocytometry confirmed that most of the Thy-1 positive cells were of the L3T4 phenotype (Table 2).

Specificity of the cellular and humoral response to VEE virus was evaluated at various times post-infection using VEE, western equine encephalitis (WEE) and eastern equine encephalitis viruses (EEE) (Fig. 1). Lymphoproliferative assays were done to evaluate mice primed to any one virus. For VEE virus primed cells the antigen stimulated proliferative response was specific for VEE virus. The maximum response occurred at day 14, along with a cross-reactive response to EEE virus. At day 54 post-immunization there was a good memory response to VEE virus, but the EEE response of the cells had disappeared. Th cells in the assay did not respond to WEE virus throughout the entire period of observation. The response of Th cells from EEE and WEE immunized mice was virus specific (Fig. 1).

When sera from all mice used in the proliferation studies were examined by ELISA, the results paralleled those seen in the proliferation assays (Fig. 2). There was a significant homologous antibody response to all viruses used for immunization with a low level cross reactivity seen between EEE and VEE viruses. The WEE virus B cell response was virus-specific, and antibodies in mice immunized with EEE or VEE virus did not cross react with WEE virus.

To analyze further the blastogenic response among more closely related alphaviruses, VEE subtype viruses were used to stimulate VEE TC-83 virus-primed cells (Table 3). The magnitude of the Th cell response correlated well with the B cell antigenic relatedness except for Everglades virus (subtype II). Everglades virus is however, neutralized by antisera prepared to the purified E2 glycoproteins of TC-83 (IA) and PTF-39 (IB) viruses (Kinney et. al., 1983).

Elevated levels of IL-2 were used as an indication of antigenic stimulation of Th cells (Table 4). Supernatants from proliferation assays were harvested 72 hrs. after antigenic stimulation and assayed for IL-2 activity with CTLL-2 cells. Levels of IL-2 were elevated only when virus-primed T cells were stimulated with homologous virus. In general, there was a direct correlation between a positive lymphoblastogenic response and elevated levels of IL-2.

II. The immune response of equines to immunization with TC-83 and recombinant vaccinia/VEE TC-5A viruses.

A. Humoral responses to immunization and virus challenge. A time line showing the immunization and challenge schedule for equines immunized with TC-83, wild-type vaccinia, and the vaccinia recombinant TC-5A are shown in Fig. 3. All horses were initially immunized on December 21, 1988. Four horses were reimmunized with TC-5A on March 22, 1989; 91 days after the initial immunization. All animals were challenged with virulent VEE virus strain 71-180 on April 12, 1989; 112 days after the initial immunization.

The immune response of horses to immunization with TC-83 and recombinant vaccinia virus TC-5A prior to virulent virus challenge has been analyzed in detail. Horses were immunized with 1×10^8 plaque forming units (PFU) of wild-type or recombinant vaccinia viruses by intradermal route. All animals given TC-83 or recombinant TC-5A developed neutralizing and ELISA reactive

antibodies after the primary immunization (Table 5). Serum from both horses (animals No. 70 and 182) which received TC-83 vaccine had very high ELISA titers and neutralization titers of 64. The four horses immunized with TC-5A (Nos. 14, 77, 134, and 160) developed low ELISA antibody titers and neutralization titers of 8 to 16. Neutralizing antibody titers of sera from these same horses to the virulent VEE virus 71-180 and vaccinia virus were determined (Table 6). Sera from the TC-83 immunized horses reacted with VEE virus 71-180 at a lower titer than they did with TC-83 virus (Table 5) and did not neutralize vaccinia virus. Antibodies in sera from the two horses immunized with vaccinia virus (Nos. 84 and 86) neutralized only vaccinia virus. Vaccinia antibody neutralization titers of sera from the TC-5A immunized horses ranged from 64 to $\geq$ 128 and did not neutralize the equine virulent VEE virus. Following secondary immunization with TC-5A, all horses developed significantly higher ELISA and neutralization antibody titers indicating that the primary immunization had sensitized the animals to VEE virus antigen (Tables 5 and 6).

When kinetics of the equine immune response following immunization was analyzed it was observed that the two TC-83 immunized animals had VEE reactive antibodies seven days after immunization (Fig.4). Antibodies to vaccinia virus were also first detected in sera from animals immunized with wild-type vaccinia or TC-5A viruses on day seven (data not shown). Seven days following secondary immunization with TC-5A recombinant virus, antibodies to VEE were demonstrated in all animals. By day 14 ELISA titers had reached levels of 6,000 (Fig.4).

The cross reactivity of antibodies in sera of horses immunized with VEE-TC-83 and TC-5A with other subtype VEE viruses, WEE, and EEE viruses is shown in Table 7. Horse No. 70 immunized with TC-83 virus was negative to all alphavirus antigens prior to immunization however, horse No. 182 was positive to both WEE and EEE viruses by ELISA. This animal had tested negative to VEE, WEE, and EEE by serum dilution neutralization prior to TC-83 immunization. After TC-83 immunization, serum from horse No. 182 reacted by ELISA with VEE subtype viruses I to IV. Animal 70, initially negative to all alphaviruses tested, reacted with all of the subtype VEE viruses tested and EEE virus. The four horses immunized with recombinant virus TC-5A which were negative by ELISA and neutralization prior to immunization were positive to all subtype VEE viruses following immunization. Thus, the cross-reactivity of ELISA antibodies following immunization with the recombinant antigen and TC-83 were similar. Horse No. 134, whose sera was positive by ELISA to WEE prior to immunization with TC-5A, developed antibodies to EEE virus after immunization.

All of the horses were challenged with 10,000 plaque forming units of virulent VEE 71-180 virus and observed 21 days. Antibody titers to VEE virus after challenge remained stable in the TC-83 immunized animals (Table 5). This indicated TC-83 immunization provided a solid immunity to virus challenge and that insufficient antigen was produced following challenge to stimulate the immune response. The antibody titers in horses immunized with TC-5A showed modest increases following challenge (Table 5). The two horses which received wild-type vaccinia virus (Nos. 84 and 86) developed

ELISA reactive antibodies by the sixth day following challenge however, their sera did not neutralize VEE virus. When kinetics of the antibody response to VEE was examined in detail it was determined that the ELISA antibody titers from the TC-83 and TC-5A animals were stable over the 21 day period following virulent virus challenge (Fig.4).

B. Cell mediated responses to immunization and virus challenge. Equine bloods were collected in heparin and the peripheral blood mononuclear cells (PBMC) separated over ficoll-paque. Blastogenic assays were set up with 2.5 X 10^8 cells using purified virus at a concentration of 1 microgram per well to stimulate the lymphocytes. Assays were incubated at 37C in 5% CO_2, pulsed with ^{3}H-thymidine and harvested at different time intervals over a three to five day period.

Representative proliferative responses to homologous and heterologous alphavirus are shown in Table 7. The CPM data have been converted to stimulation indices for comparative purposes. PBMC from VEE/TC-83 virus immunized horses were positive after the primary immunization. Two of the four vaccinia recombinant TC-5A immunized horses (Nos. 77 and 134) were positive after a single inoculation of virus. After secondary immunization with TC-5A, horse No.14 became positive however, horse No. 160 did not convert until challenged with virulent virus. Both of the vaccinia control horses showed positive proliferative responses on day six following virus challenge. Proliferation results with VEE virus 71-180 had a

high correlation with the TC-83 virus stimulation confirming the close antigenic relationship between the two viruses. Heterologous responses to WEE virus suggested either cross-reactive Th-cell epitopes are involved, or that the horses had been exposed to WEE virus some time prior to their immunization with VEE virus antigens.

III. Challenge of equines immunized with recombinant VEE/VAC, VEE/TC-83, and wild-vaccinia virus with equine virulent VEE/71-180.

A. Clinical observations. Animals immunized with the recombinant vaccinia/Venezuelan equine encephalitis virus (TC-5A), attenuated TC-83 vaccine and wild-type vaccine virus were challenged with 10,000 PFU of equine virulent VEE/71-180 virus. Two horses (Nos. 84 and 86) immunized with wild-type vaccinia virus, seronegative to VEE virus, developed elevated temperatures which they sustained until day 6 when they were euthanized (Fig. 5D). Virus was isolated from the blood of these nonimmune animals on days 1 through 3 PI (Fig. 6). Platelet and hematocrit values for these two horses were decreased and the animals were leukopenic (Fig. 7D, 9D) Clinical observations of all of the horses challenged with VEE/71-180 virus are summarized in Table 10. The two nonimmune horses, animals No. 84 and 86, became depressed and remained off food from day 1 until the morning of the third day when their fever had subsided. At this time they were more responsive however, by the afternoon of the third day the fever had returned as did the depression. By the fourth day, these two horses were eating and drinking very little

and obviously ill. On the morning of the fifth day, the horses were reluctant to move, were ataxic, stopped eating, drinking or moving. These animals showed typical clinical signs of VEE virus encephalitis and continued to clinically decline until they were euthanized on day 6. Four animals which received the VEE/VAC vaccine TC-5A (horses No. 77, 160, 14, and 134) and two which were immunized with attenuated TC-83 virus (horses No. 70 and 182) did not have a viremia, showed no hematological changes and remained clinically well throughout the 21 days following inoculation with equine virulent VEE virus (Fig. 5, 7, 9; Table 10).

B. Histopathology. Histopathologic examination was done on tissues from horses immunized with the VEE/VAC recombinant TC-5A, TC-83 and wild-type vaccinia viruses. Hematoxylin and eosin stained sections from brain (8 sections for each animal: frontal, parietal and occipital cortex, thalamus, pons, hippocampus, cerebellum and medulla) were assessed and categorized without knowledge of which experimental group each animal belonged. Histologic changes were assessed qualitatively, and where possible, quantitatively (Table 11). Mares No. 84 and 86, which were vaccinated with wild-type vaccinia virus prior to challenge, were sacrificed on day 6 following challenge. These two animals had a severe meningoencephalitis characteristic of VEE virus infection. Both animals had widespread perivascular cuffs, composed exclusively of lymphoid cells; hyperemia and focal hemorrhage; endothelial swelling; focal glial nodules and diffuse areas of gliosis, some with central necrosis, frequently in myelinated areas; mild lymphoid

meningitis. Mare No. 86 had occasional thrombi in small venules. Neurons were generally spared, except when located near areas of gliosis and necrosis. The glial nodules were relatively small (.1 - .3 mm), and no large areas of necrosis were observed. Neutrophils were not generally part of the inflammatory reaction, nor was vasculitis a prominent feature.

The other six mares, sacrificed between days 20 and 22 after challenge, had very mild or infrequent lymphoid meningitis or perivascular cuffing. These changes were most obvious in mares No. 70 and 160. Such lesions are unlikely to be of clinical significance and have been described to occur transiently between 5 and 9 days after inoculation with TC-83 vaccine. The choroid plexus was not observed in sections of mares No. 70 and 160.

Animals were evaluated by serial bleedings to monitor serum biochemical changes associated with immunization and subsequent challenge with a virulent strain of VEE/71-180. Blood samples were collected weekly following primary and secondary immunization and then daily post challenge (P.C.) on days 1-10 and again on days 14 and 21. Samples were evaluated for the following parameters: sorbitol dehydrogenase (SDH), aspartate aminotransferase (AST-SGOT), creatinine kinase (CK-CPK), glucose amylase and lipase. Prothrombin times were also monitored. Since alamine aminotransferase activity (ALT-SGPT) is virtually nonexistent in equids, its use is not indicated in this species. SDH is liver specific in the horse.

Animals were euthanized on day 6 P.C. (vaccinia controls) or on days 21, 23 P.C. (all others) and selected tissues were submitted for histopathological examination. With the exception of lesions in

the brains of control animals, histopathological examination was unremarkable. Prothrombin times were within normal limits. Analysis of serum for the above parameters was also unremarkable except in the case of the two control animals. Horse No. 84 exhibited elevations in SDH and AST (Day 6 P.C.) while horse No. 86 showed increases in SDH (Days 3-6 P.C.), AST and CK (Days 5-6 P.C.). These changes are consistent with mild myocellular injury (Horse No. 86) and hepatocellular damage (Horse No. 84 and 86) following peripheral challenge with a virulent VEE/71-180.

IV. Construction of an infectious VEE virus cDNA clone.

Understanding viral pathogenesis at the molecular level is important because it is critical in the development of safer and more effective attenuated vaccines. Much of the progress in understanding attenuation has resulted from characterization of virus strains of altered virulence. Detailed nucleotide and amino acid sequence comparisons between pairs of virulent and attenuated strains has often been frustrated by the presence of numerous coding and noncoding nucleotide differences. Thus, the assignment of specific mutations to a particular phenotype is difficult. Assignment of particular mutations to specific phenotypes of RNA viruses has been greatly improved by the construction of full-length cDNA clones. Infectious progeny virus with specific genetic characteristics can be produced by transcribing the cDNA and using the viral RNA to transfect host cells. The availability of complete cDNA clones of the virus genome allows construction of strain

pairs that are isogenic except for defined nucleotide substitutions. Such procedures have facilitated the study of a number of virus phenotypes, including the pathogenesis phenotype. In this report, the power of this approach has been applied to the analysis of VEE virus to identify genetic foci which modulate the virulence of the virus in weanling mice.

A full-length cDNA clone designated pVE/IC-92 was constructed from subgenomic clones of VEE TC-83 and TRD viruses previously described. (Fig 10). The nucleotide sequence of pVE/IC-92 has been determined in its entirety and is identical to that of TC-83 (Kinney et al., 1989). The subgenomic clones used to construct pVE/IC-92 are shown in Fig. 11. Errors detected in the nucleotide sequence of the TC-83 clones were corrected or spliced out using cognate regions of TRD clones which contained sequences identical to TC-83.

Critical restriction endonuclease splice sites used in construction of pVE/IC-92 are shown in Fig.11. The clone is bounded in pUC18 at the 5'- end by an XbaI site and the T7 RNA polymerase promoter. At the 3'-end of the clone is located a 107- base pair synthetic DNA fragment (pVE/IC-3) which contains the 3'- end of the genome, a poly adenylic acid tail of 25 residues, and a unique MluI site which upon digestion yields the 3'-poly A tail free of extraneous nucleotides. XL-1 Blue E. coli cells have been transformed with the pVE/IC-92 clone in order to amplify the clone for genetic analysis and transcription.

Purified plasmid pVE/IC-92 was analyzed by restriction endonuclease mapping to confirm the presence of restriction sites used to ligate the subgenomic fragments. The SalI, BglII, SstI, ApaI, and SphI sites used

to assemble clone pVE/IC-92 were present (Fig.12). Digestion of the clone with restriction endonuclease MluI opened the clone at the 3'-end as predicted from the sequence (Fig.13).

RNA transcripts synthesized from the cDNA clone in vitro using T7 RNA polymerase were infectious for CV-1 cells using Lipofectin to facilitate uptake of the RNA. Virus produced in CV-1 cells following transfection produced typical TC-83 size plaques on Vero cells which were 0.5-1.0 mm in size.

To examine the effect of different amino acid substitutions on the neurovirulence of VEE virus, a number of recombinant viruses were constructed (Fig.13). To accomplish this, restriction fragments from TRD clone TRD-1 were used to replace the corresponding restriction fragments in clone pVE/IC-92. These recombinant cDNA clones contain a complete cDNA copy of TC-83 virus inserted downstream from a T7 RNA polymerase promoter from which infectious RNA can be transcribed in vitro. RNA was transcribed from the recombinant clones and transfected into CV-1 cells and the cells were incubated at 37 C under liquid medium to rescue infectious virus. The titers of the rescued viruses were between 10^6 - 10^8 PFU/ml.

The constructs to be tested for their neurovirulence are illustrated schematically in Fig.13. The restriction sites used to construct the hybrid genomes are shown together with the region of the genome which originates from TRD virus RNA. In all cases, the recombinants possess the nonstructural region from pVE/IC-92 and only the glycoprotein regions or portions of the glycoprotein region derived from clone TRD-1. The plaque morphology of the recombinant viruses on Vero cells was first

examined (Table 14). Plaque of TRD virus on Vero cells are 3.0-4.5mm in size with clear edges whereas TC-83 plaques are 1.0 mm in size with rough edges giving a star like appearance. Virus from the infectious clone pVEIC/92 produced small TC-83 like plaques. Recombinant 106 which contained the E1 TRD virus amino acid Leu at position 161 in E1, recombinant 104 which contained Lys and His at E2 positions 7 and 85 respectively, and recombinant 107 which contained the Thr at position 120 all produced small TC-83 like plaques. Only recombinant 102 which contained the entire structural gene sequence of TRD and recombinant 105 which contained Thr, Val, and Thr at positions 12, 192, and 296 in E2 gave larger plaques. This suggests that the amino acid changes in E2 are more important in determining plaque size than those in E1 and that more than one amino acid change is involved in the plaque size phenotype.

The neurovirulence of each of the recombinant viruses, the parental virus pVE/IC-92, TC-83 and TRD viruses are now being tested in 3-to 4-week-old mice inoculated i.p. with 10^6 - 10^2 PFU of each virus.

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VI. Publications

KINNEY, R.M., B.J.B. Johnson, J.B. Welch, K.R. Tsuchiya and D.W. Trent (1989). The full-length nucleotide sequences of the virulent Trinidad donkey strain of Venezuelan equine encephalitis virus and its attenuated vaccine derivative, strain TC-83. Virology **170**, 19-30.

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Table 1. Phenotyping of nylon wool passaged T-cells from normal and alphavirus primed C3H mice after depletion with specific antisera.

		T-cell depletion		
		Pre-proliferation	Post-proliferation	
Treatment[a/]	% cytotoxicity	VEE TC-83[b/]	Sindbis	VEE TC-83
Diluent	5	97,791 ± 13,626	34,054 ± 2,197	44,859
Anti-Thy-1	60	3,046 ± 3,118	No data	1,879
Anti-Lyt-1.1	49	3,250 ± 992	<1000	7,366
Anti-Lyt-2.1	29	92,457 ± 5,098	20,132 ± 2,467	36,596

a/ Plus complement.

b/ Results shown are from stimulation of virus-primed-T-cells with homologous virus only. Similar treatment of normal, unprimed T-cells gave negative lymphoproliferation values. Results are c.p.m. of ^{3}H-thymidine incorporation after complement treatment.

Table 2. Phenotypic analysis of nylon wool passaged lymphocytes by flow cytometry.

		Percent Staining Lymphocytes	
Antibodies[a/]	Cell Type	Normal[b/]	VEE TC-83[c/]
Anti-Thy-1	All T-cells	65	74
Anti-L3T4	All Th-cells	43	50
Anti-Lyt-2	All Tc-cells	21	20
Anti-IgG	B-cells	No data	20

a/ Thy-1, Lyt-2, and IgG antisera were conjugated to flourescein isothiocyanate and L3T4 antisera to pycoerythrin.

b/ Lymphocytes from uninoculated C3H mice were labeled and phenotyped following nylon wool chromatography.

c/ Lymphocytes were processed from mice four weeks after inoculation with VEE TC-83 virus and incubated <u>in vitro</u> with homologous virus.

Table 3. Stimulation of VEE TC-83 virus-primed nylon wool passaged T-cells with VEE subtype viruses.

Virus[a/]	Subtype	Lymphoproliferation	%
TC-83	IAB	66,376 ± 3,349[b/]	100
PTF-39	IAB	94,660 ± 5,359	142
P676	IC	45,344 ± 1,897	68
3880	ID	27,545 ± 2,411	41
Mena II	IE	36,862 ± 2,986	56
Everglades	II	71,327 ± 6,271	93
Mucambo	III	22,130 ± 1,209	33
Pixuna	IV	23,781 ± 3,274	36

a/ Stimulator splenocytes not preinfected; purified virus (1 ug) added to wells at time of assay.

b/ Values are c.p.m. of adjusted means ± associated S.E.

Table 4. Comparison of the lymphoblastogenic response and interleukin-2 (IL-2) production after homologous and heterologous stimulation of T-cells from mice primed with purified virions.

		Purified virus primed T-cells		
Virus	Assay[a/]	TC-83	EEE	WEE
TC-83	Blast.	29,896 ± 1,889	<1000	1,515 ± 1,635
	IL-2	7,432 ± 829	<500	<500
EEE	Blast.	5,351 ± 2,731	25,471 ± 1,473	<500
	IL-2	<500	12,778 ± 1,404	<500
WEE	Blast.	<1000	<1000	24,714 ± 1,238
	IL-2	<500	<500	4,492 ± 658

a/ Blastogenesis of nylon wool purified lymphocytes. IL-2 assay was incorporation of ^{3}H-thymidine by CTLL-2 cells after 18 h treatment with blastogenesis supernatant.

Table 5. Serological reactivities of horse vaccinees with TC-83 virus.

Horse	Immunogen	Test	Serum Sample[a/] 1⁰	2⁰	Post-Challenge
70	TC-83	EIA	25600	n.a.[b/]	3200
		N	≥1024	n.a.	128
182	TC-83	EIA	25600	n.a.	12800
		N	≥1024	n.a.	128
14	TC-5A	EIA	100	3200	25600
		N	8	32	128
77	TC-5A	EIA	≤100	3200	6400
		N	16	128	256
134	TC-5A	EIA	100	3200	12800
		N	8	32	64
160	TC-5A	EIA	100	25600	12800
		N	8	128	256
84	Vaccinia	EIA	≤100	≤100	1600
		N	<4	≤2	≤2
86	Vaccinia	EIA	≤100	≤100	1600
		N	<4	≤2	≤2

a/ Highest observed titers.

b/ Not applicable.

Table 6. Neutralization reactivities of horse vaccinees with equine virulent VEE virus 71-180 and vaccinia viruses.

			Serum Sample[a/]		
Horse	Immunogen	Test	1^0	2^0	Post-Challenge
70	TC-83	71-180	≥256	n.a.[b/]	256
		Vaccinia	<2	n.a.	≤2
182	TC-83	71-180	≥1024	n.a.	256
		Vaccinia	<2	n.a.	≤2
14	TC-5A	71-180	<2	32	256
		Vaccinia	64	64	64
77	TC-5A	71-180	<2	32	64
		Vaccinia	64	128	128
134	TC-5A	71-180	<2	32	128
		Vaccinia	≥128	128	256
160	TC-5A	71-180	<2	32	128
		Vaccinia	≥128	128	512
84	Vaccinia	71-180	<2	≤2	1600
		Vaccinia	≥128	64	128
86	Vaccinia	71-180	<2	≤2	1600
		Vaccinia	≥128	128	128

a/ Highest observed titers.

b/ Not applicable.

Table 7. ELISA cross-reactivity of sera from immunized equines.

Horse	Immunogen	Time[a/]	Antigens								
			IAB	IC	ID	IE	II	III	IV	WEE	EEE
70	TC-83	Pre	–	–	–	–	–	–	–	–	–
		Post	+	+	+	+	+	+	+	–	+
182	TC-83	Pre	–	–	–	–	–	–	–	+	+
		Post	+	+	+	+	+	+	+	+	+
14	TC-5A	Pre	–	–	–	–	–	–	–	–	–
		Post	+	+	+	+	+	+	+	–	–
77	TC-5A	Pre	–	–	–	–	–	–	–	–	–
		Post	+	+	+	+	+	+	+	–	–
134	TC-5A	Pre	–	–	–	–	–	–	–	+	–
		Post	+	+	+	+	+	+	+	+	+
160	TC-5A	Pre	–	–	–	–	–	–	–	–	–
		Post	+	+	+	+	+	+	+	–	–

a/ Time pre- or post-primary (TC-83) or secondary (TC-5A) immunization.

Table 8. Equine Th-cell stimulation index to various VEE virus vaccines and heterologous alphaviruses.

Horse	Immunogen	Time[b/]	Antigen[a/] TC-83	71-180	WEE	EEE
70	TC-83	1^0	4.5			
		C	4.4	3.5	1.0	1.0
182		1^0	10.0			
		C	3.0	2.5	2.7	1.0
14	TC-5A	1^0	1.0			
		2^0	2.4	3.2		
		C	13.0	16.0	1.0	1.0
77		1^0	2.8			
		2^0	2.8	4.4		
		C	3.7	3.1	5.8	1.0
134		1^0	4.5			
		2^0	2.6	2.3		
		C	7.8	6.6	1.0	1.0
160		1^0	1.0			
		2^0	1.0	1.0		
		C	2.2	2.2	1.0	1.0
84	Vaccinia	1^0	1.0			
		2^0	1.0	1.0		
		C	1.9	2.9		
86		1^0	1.0			
		2^0	1.0	1.0		
		C	2.2	3.3		

a/ Stimulation index is $\frac{\text{C.P.M. test (Lymphocytes + Virus)}}{\text{C.P.M. Control (Lymphocytes + Diluent)}}$

A value of 1 indicates no stimulation.

b/ Results are from the day with the highest values, usually 2-3 weeks post-primary inoculation (1^0), 4 days post-boost (2^0), and 14 days postchallenge (C).

Table 9. Equine blood chemistries following challenge with equine virulent VEE virus 71-180.

Test	Range Observed	Normal Values
Glucose	51-156	67-135 mg/L
Creatine Phosphokinase	6-182	34-390 Su/L
Sorbitol Dehydrogenase	0-16	0-9 U/L
SGOT	184-366	204-250 U/L
Amylase	<21-<30	≤28 U/L
Lipase	0.1-0.2	0.9-1.5 U/ml

Table 10. Equine Clinical Course -- VEE IB

Immunization & Challenge	Animal I.D.	VEE IB -- Post Challenge Clinical Course
Vaccinia TC-5A (I.D.)	77	No Clinical Abnormalities Noted
	160	No Clinical Abnormalities Noted
VEE IB (SC)	14	No Clinical Abnormalities Noted
	134	No Clinical Abnormalities Noted
Vaccinia (I.D.)	84	*1. Temperature elevated = D-1 (am) - D-2 (pm) Biphasic fever as normal D-3 (am) then increased D-3 (pm) - D-6 (pm) 2. Depressed mental status = D-1 (pm) -- D-6 (pm) 3. Anorexia = Incomplete progressing to complete by Day 4 - Day 6 4. Oliguria = anuria D-1 -- D-6 (Progressive)
VEE-IB (SC)	86	*5. Anemia + decreased platelet counts + hemoconcentration (not thrombocytopenic) 6. Ataxia with wide based stance 7. Aberrant mental status (teeth grinding, constant chewing motion) 8. Terminally somnolent - very reluctant to ambulate.
TC-83 (SC)	70	No Clinical Abnormalities Noted
VEE-IB (SC)	182	No Clinical Abnormalities Noted
	Synopsis=	Animals becamse depressed, off feed and water initially beginning on Day 1 Post-Challenge (pm). This trend continued but on Day 3 the fever subsided, the animals appeared more responsive and ate/drank that morning. By pm of Day 3 - the fever returned as did the depression. By Day 4, the animals were eating, drinking little or nothing, the depressed mental status returned. The animals became progressively reluctant to move, became ataxic and stopped eating/drinking or moving altogether - until euthanized.

Table 11. Summary of histopathologic findings in the CNS of horses immunized with recombinant vaccine TC-5A, TC-83 or wild-type vaccinia virus and challenged with virulent VEE virus.

Mare	Vaccine	Histopathologic Findings in CNS
70	TC-83	focal perivascular lymphoid infiltration - mild; cerebellum, hippocampus, thalamus
182	TC-83	meningeal lymphoid infiltration - mild
14	TC-5A	focal perivascular lymphoid infiltration - mild; pons chronic focal perivascular fibrosis - mild; cerebrum, chorioid plexus, pons (probably incidental finding)
77	TC-5A	focal perivascular lymphoid infiltration - mild; cerebrum
134	TC-5A	meningeal lymphoid infiltration -mild
160	TC-5A	meningeal lymphoid infiltration - mild; focal perivascular lymphoid infiltration - mild; cerebrum, hippocampus, pons, cerebellum
84	Vaccinia	non-suppurative meningoencephalitis - severe
86	Vaccinia	non-suppurative meningoencephalitis - severe, with thrombosis

Table 12. Coding differences in VEE virus E1 and E2 glycoprotein genes.

Strain/ Recombinant	Phenotype/ Plague Size	Residue at Position					
		E1	E2				
		161	7	85	120	192	296
pVE IC/92	0.5-1.0 mmS	Ile	Asn	Tyr	Arg	Asp	Ile
pVE IC/102	2.0-4.0 mm	Leu	Lys	His	Thr	Val	Thr
pVE/IC 103	2.0-2.5 mm	0	Lys	His	Thr	Val	Thr
pVE/IC 104	0.5-1.0 mmS	0	Lys	His	0	0	0
pVE/IC 105	1.0-1.5 mmS	0	0	0	Thr	Val	Thr
pVE/IC 106	0.5-1.0 mmS	Leu	0	0	0	0	0
pVE/IC 107	0.5-1.0 mmS	0	0	0	Thr	0	0
VEE TC-83	1.0 mmS	Ile	Asn	Tyr	Arg	Asp	Ile
VEE TRD	3.0-4.5 mm	Leu	Lys	His	Thr	Val	Thr

FIGURE LEGENDS

Fig 1. Lymphoproliferative response of (a) VEE TC-83, (b) EEE and (c) WEE virus-primed and pooled (three to six mice per virus group) NW-purified responder T cells. Stimulator cells were infected with purified viruses VEE TC-83 (solid columns), VEE Trinidad (hatched columns), WEE (blank columns) and EEE (dotted columns). Bars represent the adjusted mean counts per minute in three replicates with the standard error.

Fig. 2 Endpoint ELISA titers of mouse serum specimens collected at time intervals corresponding to the T cell assays and tested against purified viruses VEE TC-83 (solid columns), EEE (dotted columns) and WEE (blank columns). Bars represent mean values of four to six sera for each virus $\pm$ standard error.

Fig. 3 Time line indicating the days following immunization of horses with TC-83 (112 days), TC-5A (91 days after the primary immunization and 21 days following the second immunization) and wild-type vaccinia (112 days) prior to virulent virus challenge at 112 days. Control horses were euthanized on day six following challenge. The horses immunized with TC-83 and TC-5A were euthanized on day 21 after virulent virus challenge.

Fig. 4 Analysis of ELISA antibodies in equines after immunization with TC-83, TC-5A and wild-type vaccinia viruses.

Fig. 5 Body temperature of horses immunized with TC-5A (A and B), TC-83 (C) and wild-type vaccinia virus (D) and challenged with equine virulent VEE virus 71-180.

Fig. 6. Body temperatures and viremia of horses immunized with wild-type vaccinia virus and challenged with equine virulent VEE virus 71-180. Solid dots and blank columns horse 84; open dots and hatched columns horse 86.

Fig. 7 Hematocrit values of horses immunized with TC-5A (A and B), TC-83 (C) and wild-type vaccinia virus (D) and challenged with equine virulent VEE virus 71-180.

Fig. 8 Total white blood cell counts from horses immunized with TC-5A (A and B), TC-83 (C) and wild-type vaccinia virus (D) and challenged with equine virulent VEE virus 71-180.

Fig. 9 Platelet counts from horses immunized with TC-5A (A and B), TC-83 (C) and wild-type vaccinia virus (D) and challenged with equine virulent VEE virus 71-180.

Fig. 10 Strategy used for obtaining the complete nucleotide sequences of VEE Trinidad donkey and TC-83 viruses. Clones that were sequenced are indicated in open rectangles lacking the "X" symbol. Arrows indicate where primer extension RNA sequencing was done to complete the sequence. Organization of the genome is shown at the top, and the genome position in kilobases is shown at the bottom.

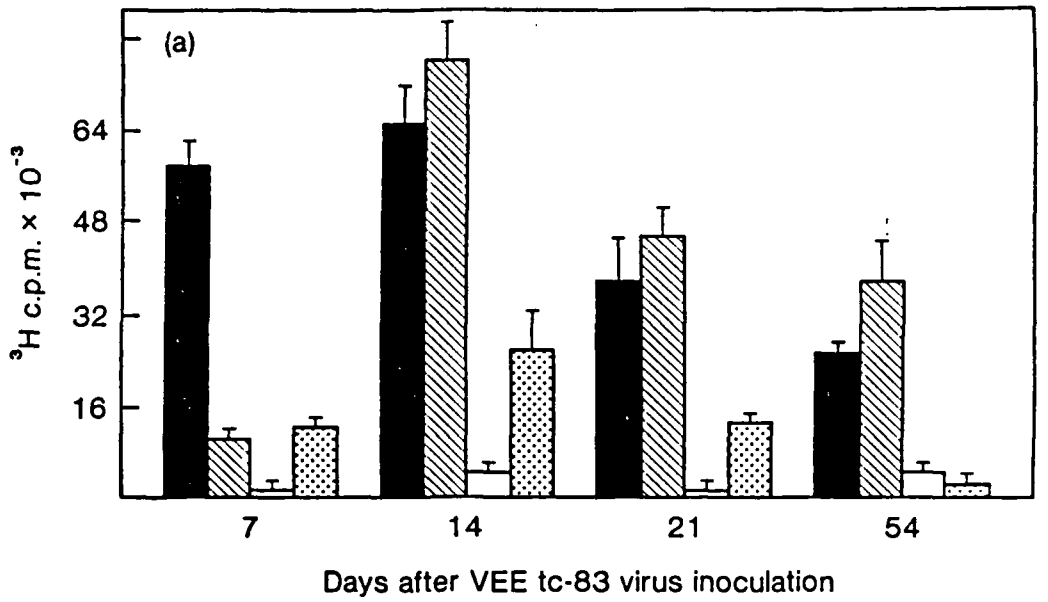
Fig. 11 Strategy for construction of a complete cDNA clone of VEE TC-83 virus by ligation of subgenomic clones of pTC-46, pTC-19, pTRD-30, pTC-9, pTRD-26 and pVE/IC-3 together at the indicated unique restriction sites.

Fig. 12 Digestion of clone pVE/IC-92 at unique predicted restriction endonuclease sites to confirm the junctions of all subgenomic cDNA clone ligations.

Fig. 13 Digestion of clone pVE/IC-92 with restriction endonucleases to confirm the integrity of cloned cDNA in the final construction of the full length gene copy of TC-83.

Fig. 14 Construction of recombinant VEE virus genomes in the glycoprotein region. Restriction fragments in clone pVE/IC-92 were replaced by the corresponding fragments from Trinidad donkey clone pTRD-1 indicated as black bars in the diagram. Changes in amino acid sequence from TC-83 to Trinidad donkey are indicated in letters above the sequence.

Fig. 1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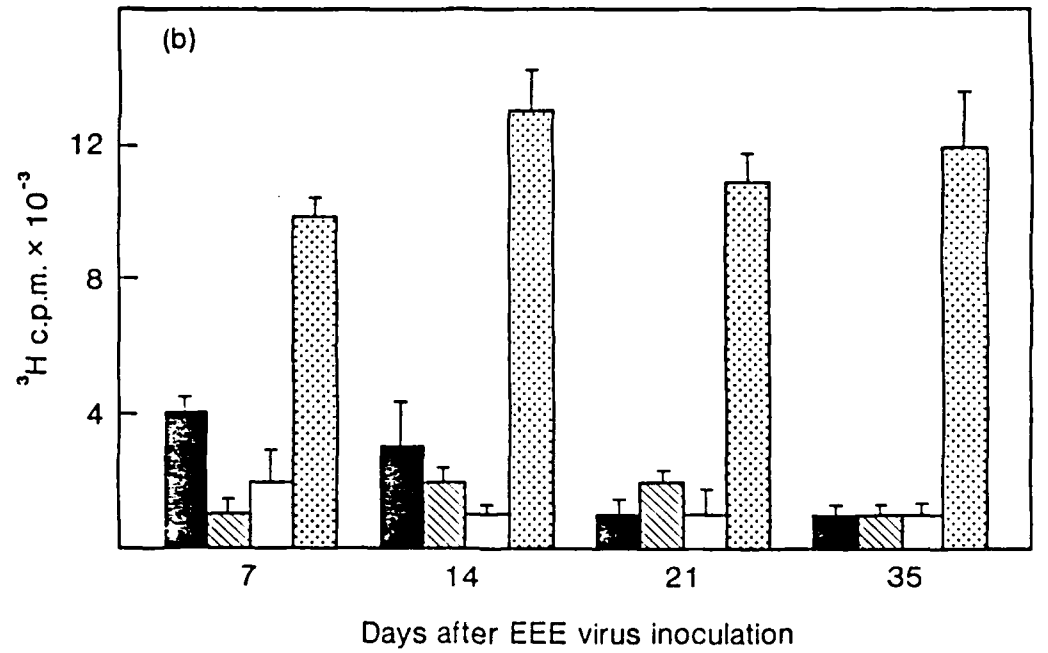
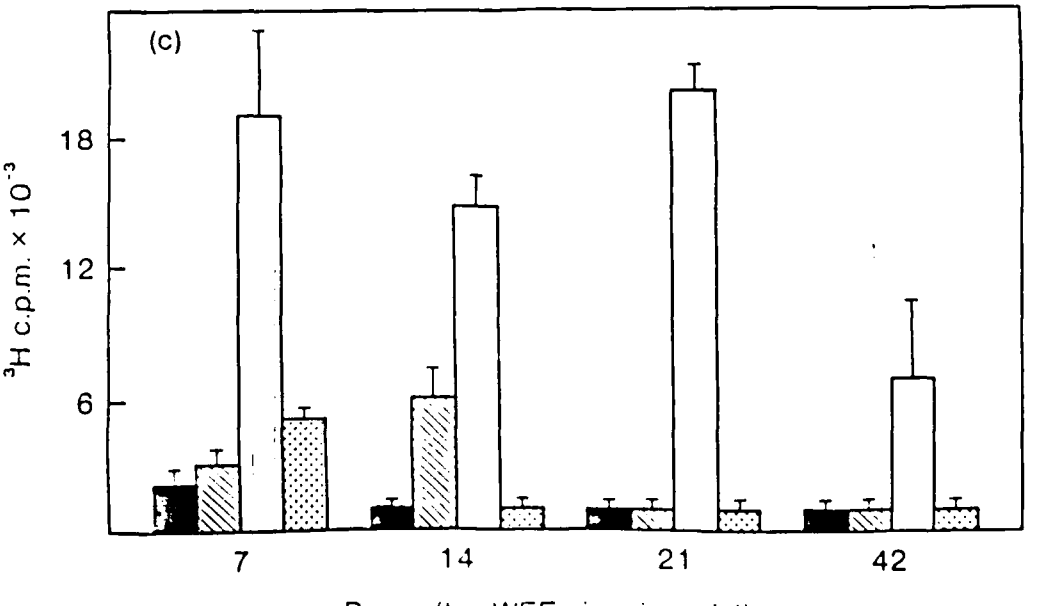
48
32
16
^{3}H c.p.m. x 10^{-3}
7
14
21
54
Days after VEE tc-83 virus inoculation


(b)
12
8
4
^{3}H c.p.m. x 10^{-3}
7
14
21
35
Days after EEE virus inoculation


(c)
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6
^{3}H c.p.m. x 10^{-3}
7
14
21
42
Days after WEE virus inoculation

Fig. 2

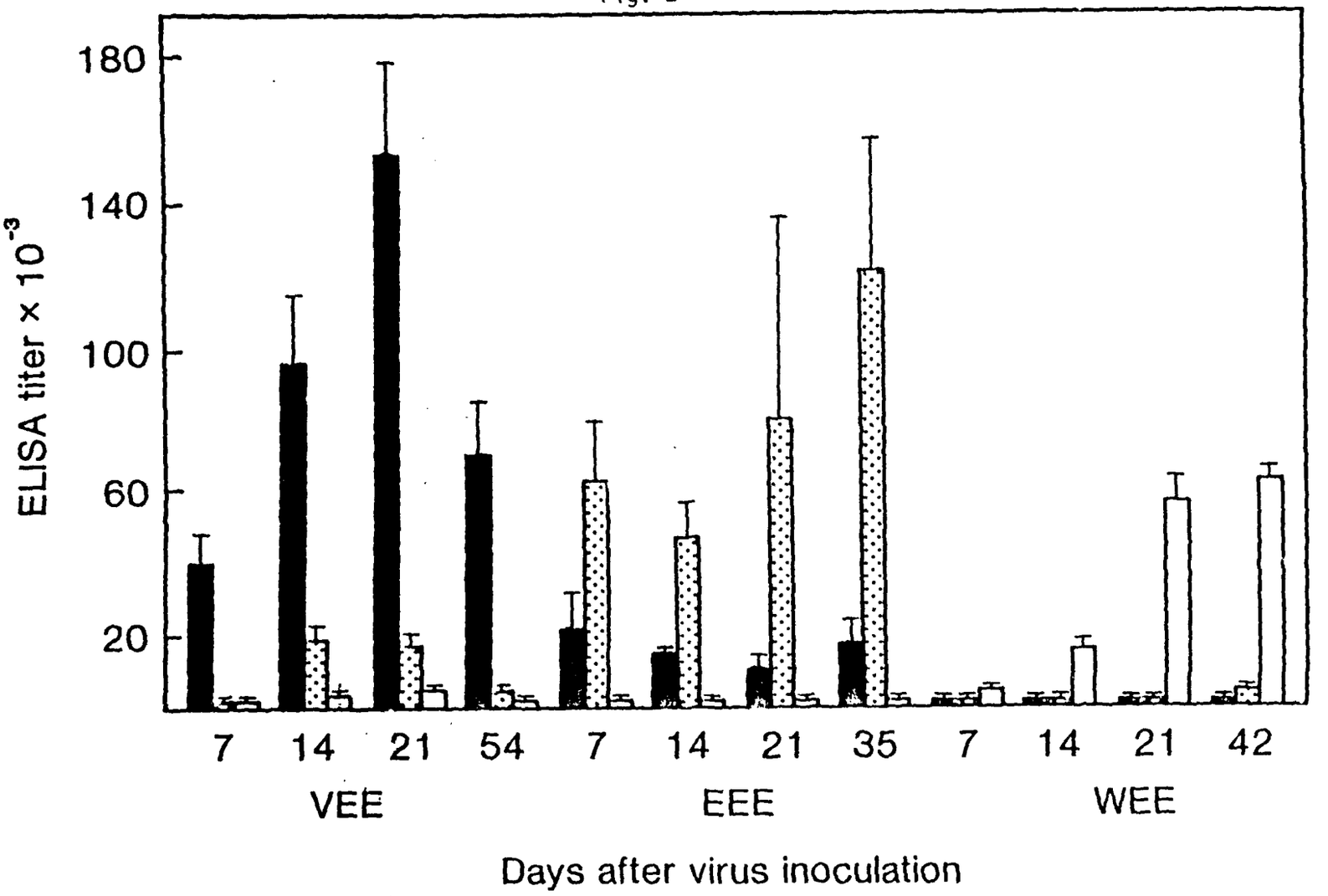
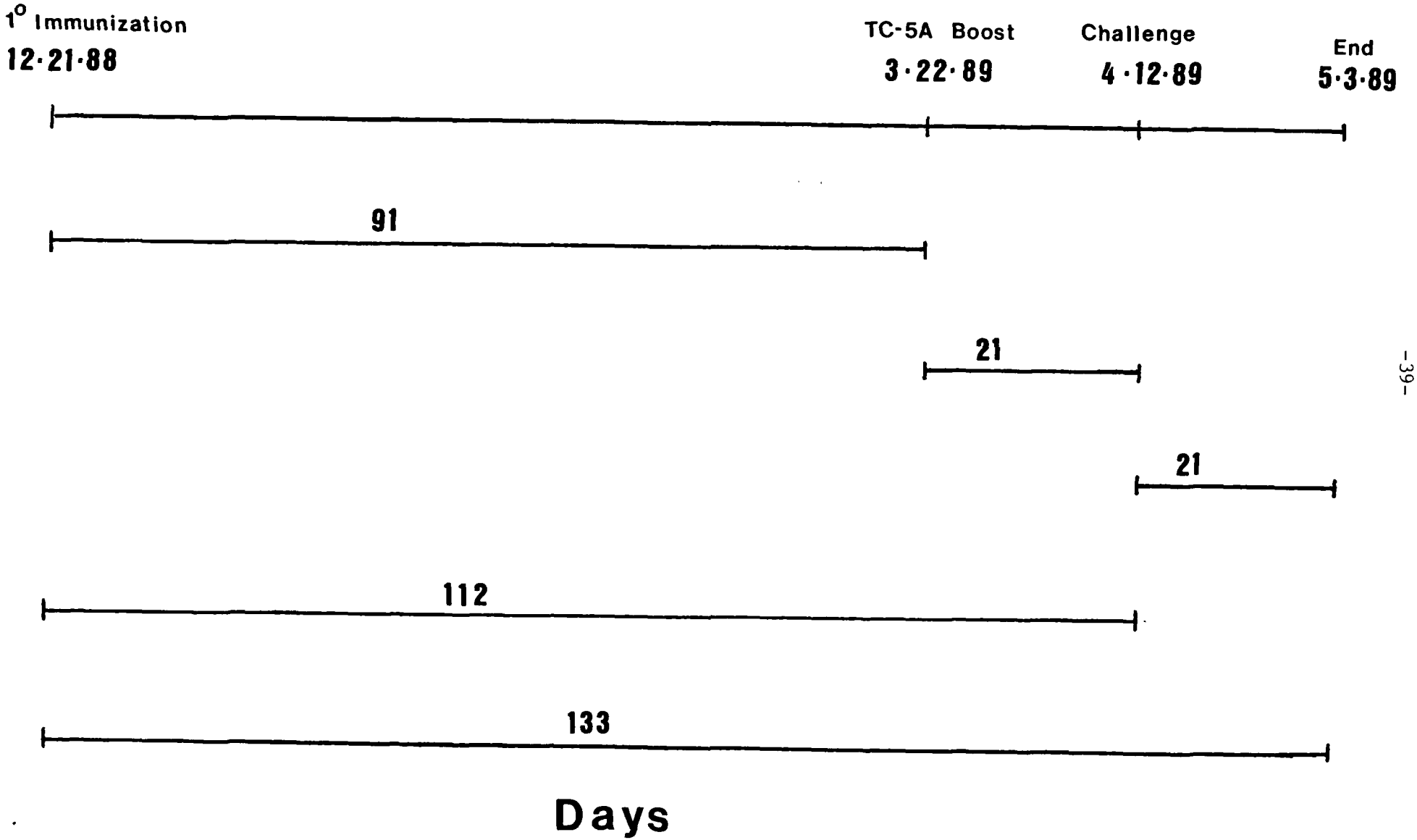

180
140
100
60
20
ELISA titer × 10⁻³
7
14
21
54
7
14
21
35
7
14
21
42
VEE
EEE
WEE
Days after virus inoculation

Fig. 3


1° Immunization
12·21·88
TC-5A Boost
3·22·89
Challenge
4·12·89
End
5·3·89
91
21
21
112
133
Days

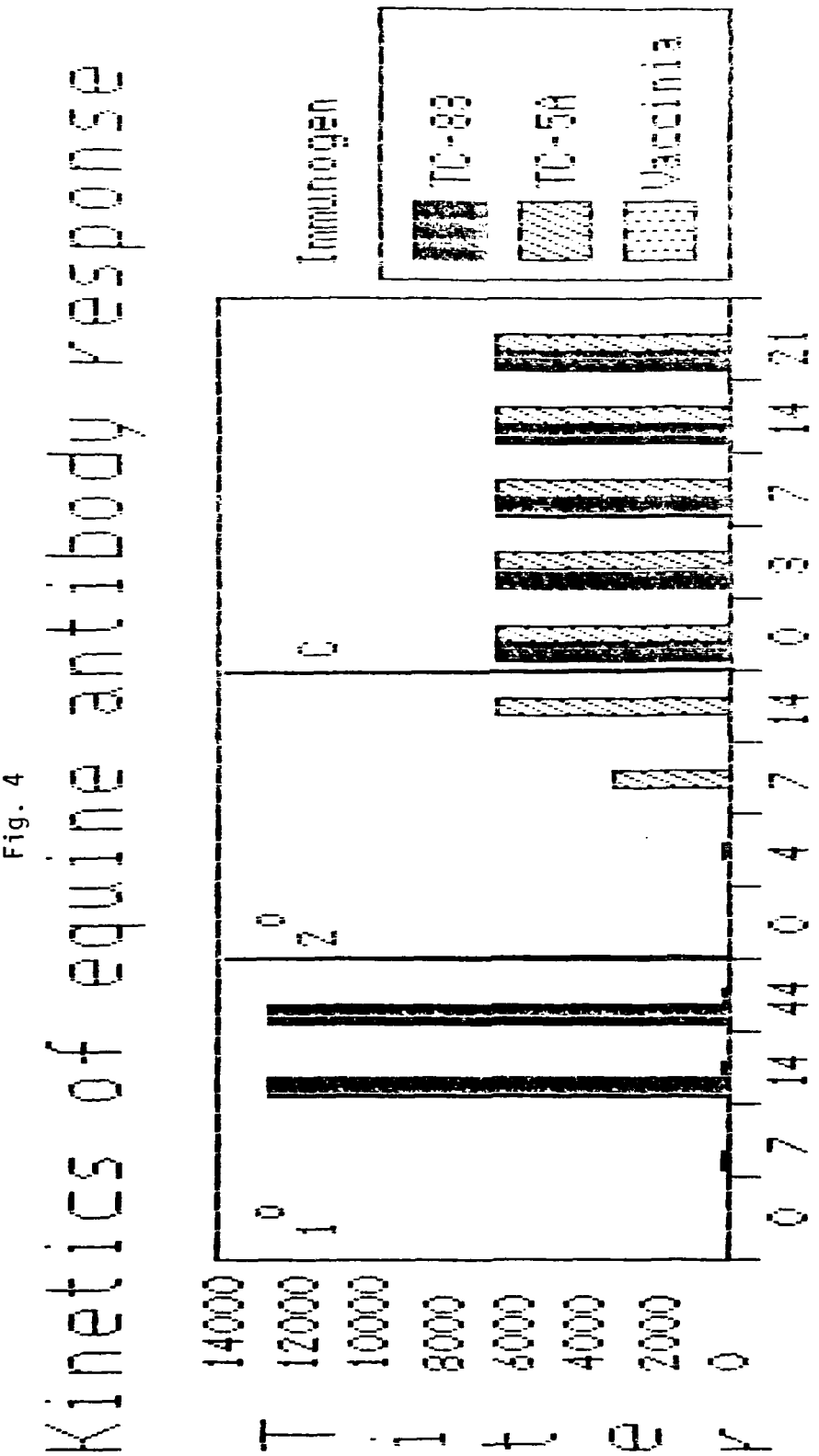
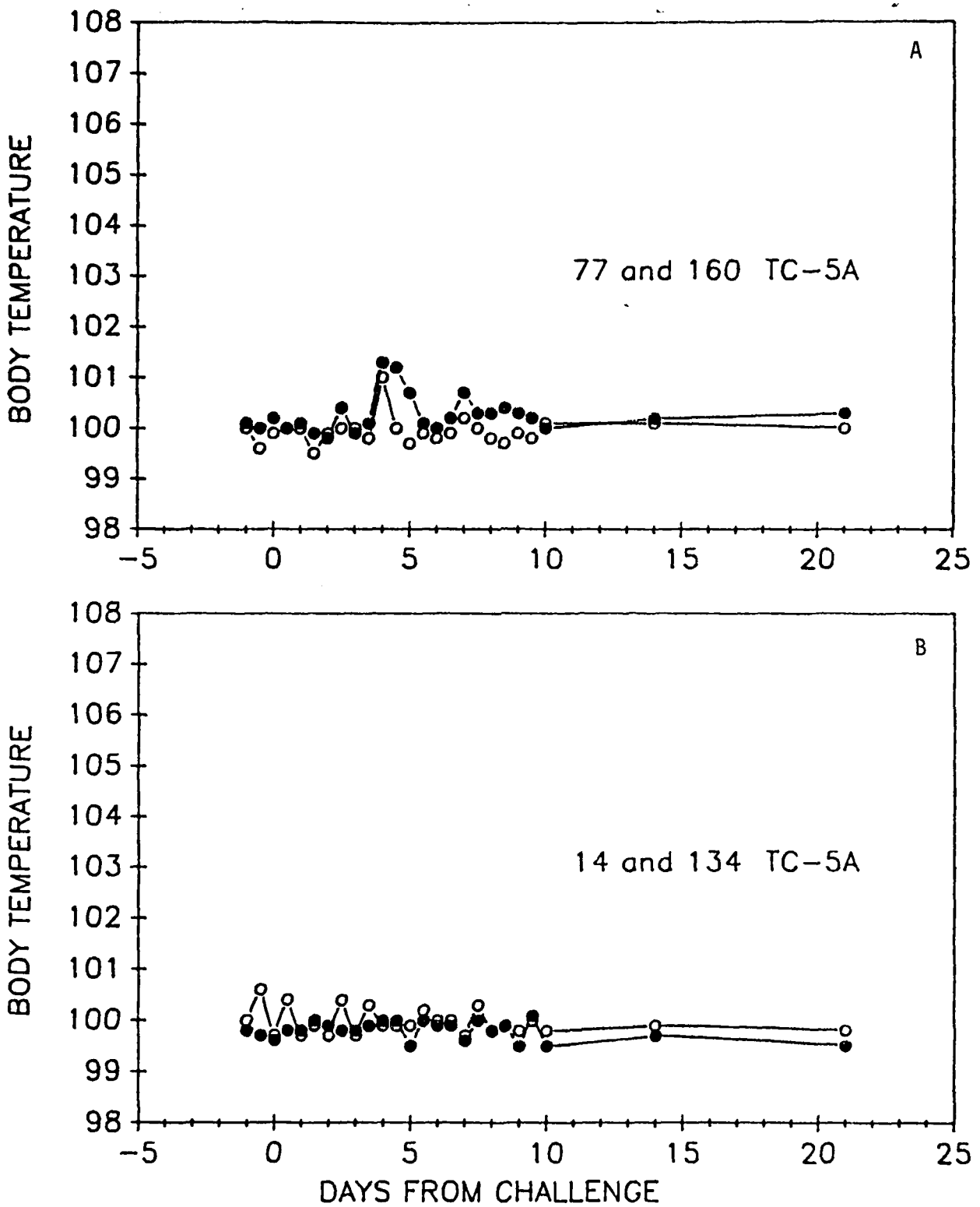

Kinetics of equine antibody response
Titer
14000
12000
10000
8000
6000
4000
2000
0
Immunogen
TC-83
TC-5M
Vaccinia
0
1
0
2
0
4
7
14
44
0
7
14
21
28
Days

Fig. 4

Fig. 5


A
77 and 160 TC-5A
BODY TEMPERATURE
108
107
106
105
104
103
102
101
100
99
98
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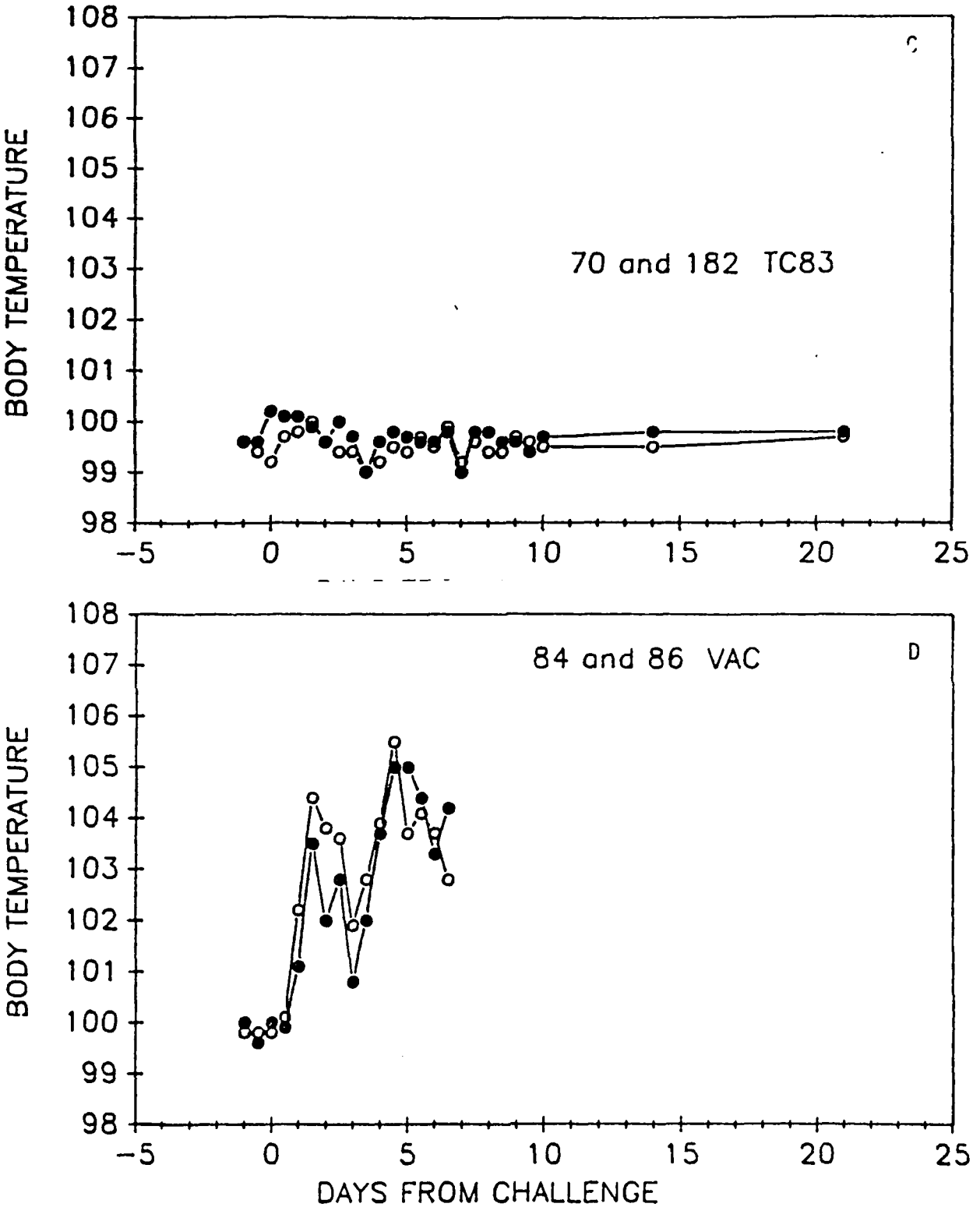
0
5
10
15
20
25
B
14 and 134 TC-5A
BODY TEMPERATURE
DAYS FROM CHALLENGE

Fig. 5


C
70 and 182 TC83
BODY TEMPERATURE
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107
106
105
104
103
102
101
100
99
98
-5
0
5
10
15
20
25
D
84 and 86 VAC
BODY TEMPERATURE
DAYS FROM CHALLENGE

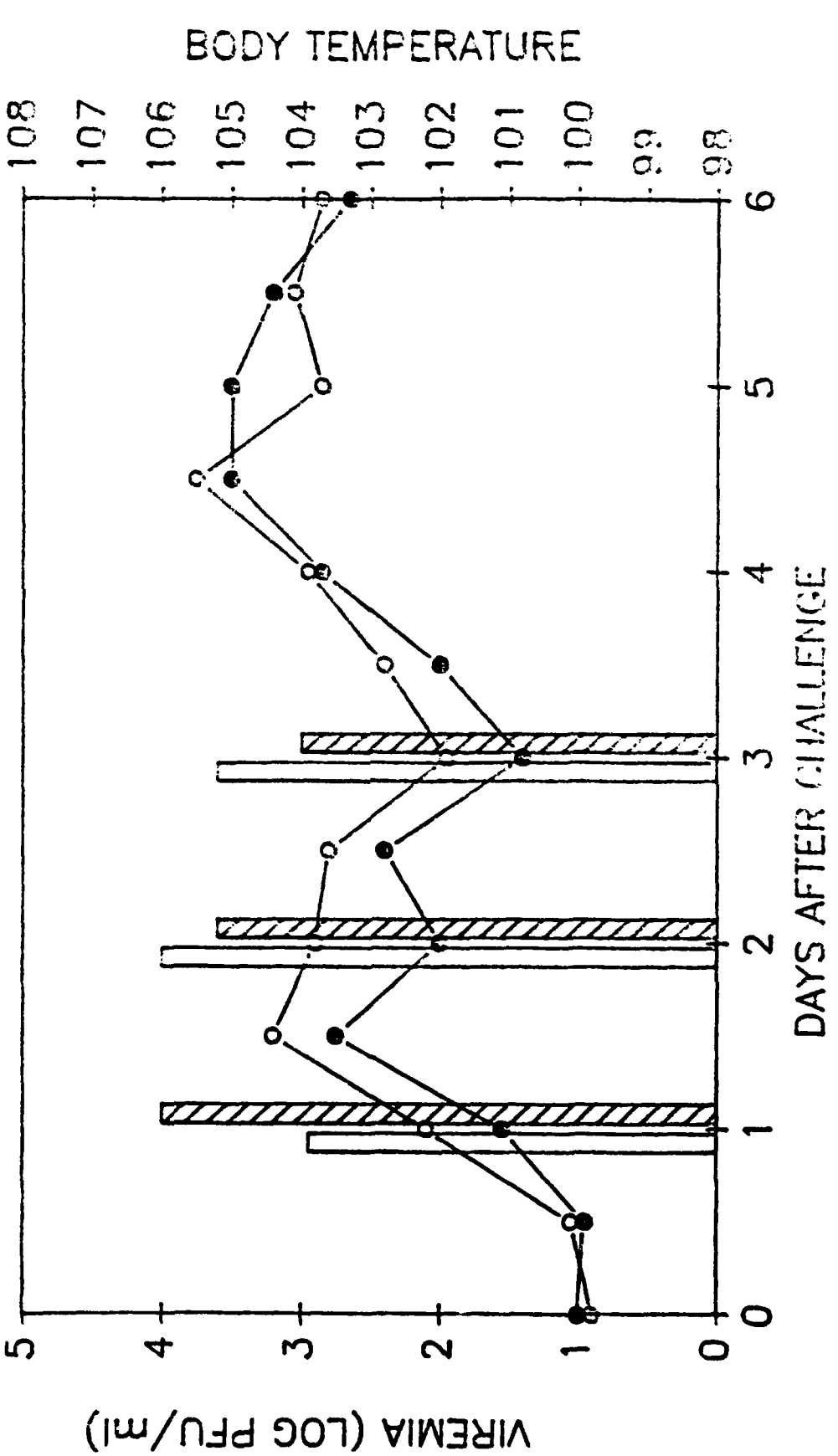
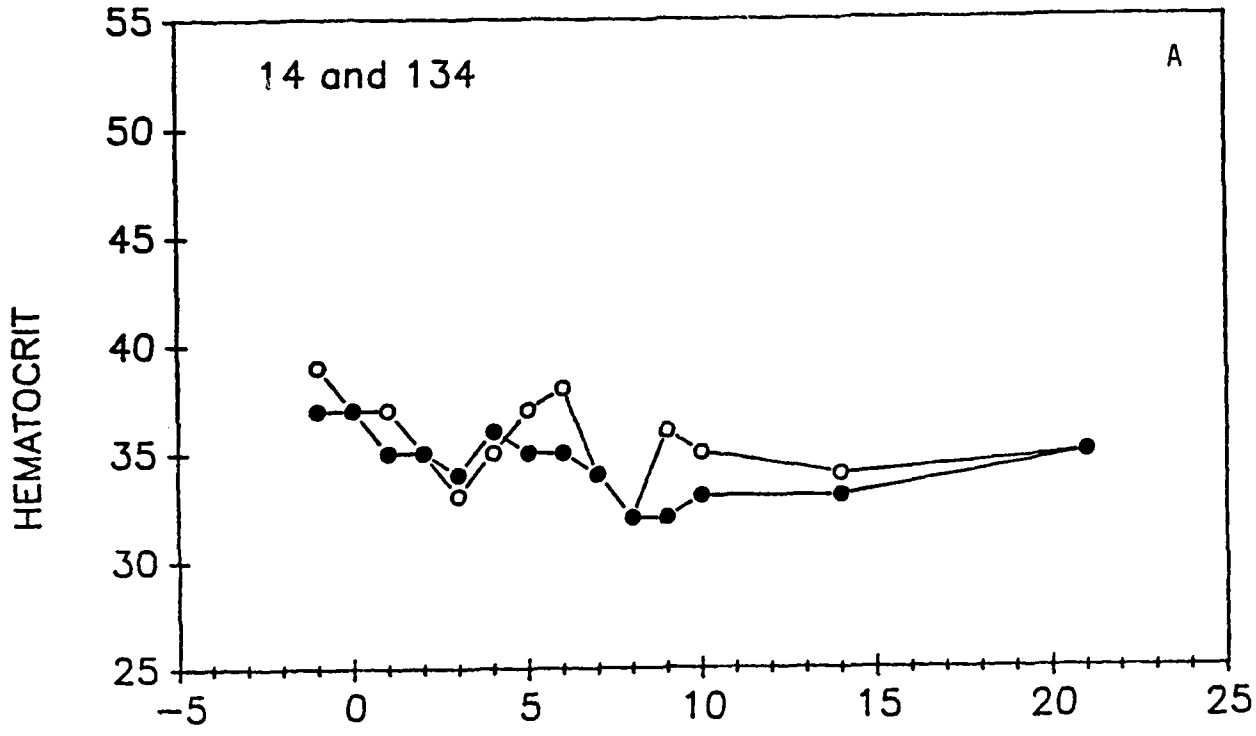

BODY TEMPERATURE
108
107
106
105
104
103
102
101
100
99
98
VIREMIA (LOG PFU/ml)
5
4
3
2
1
0
DAYS AFTER CHALLENGE
0
1
2
3
4
5
6

Fig. 6

Fig. 7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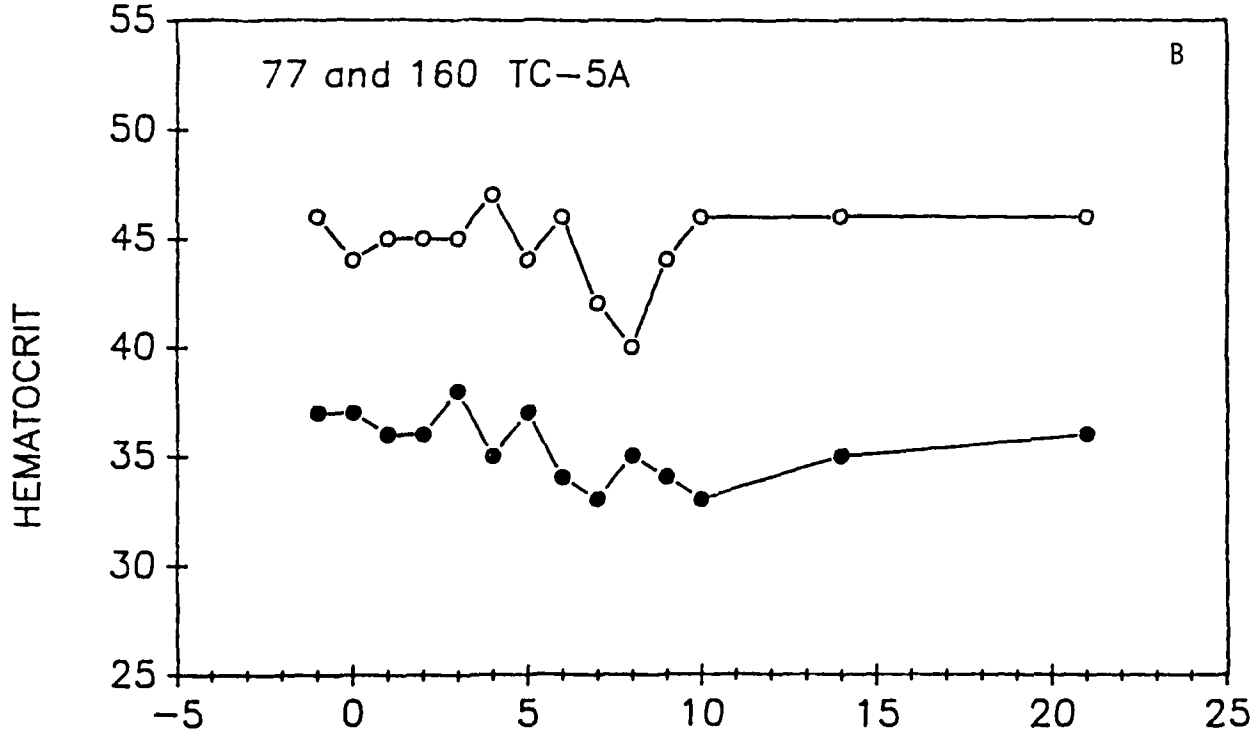
15
20
25


77 and 160 TC-5A
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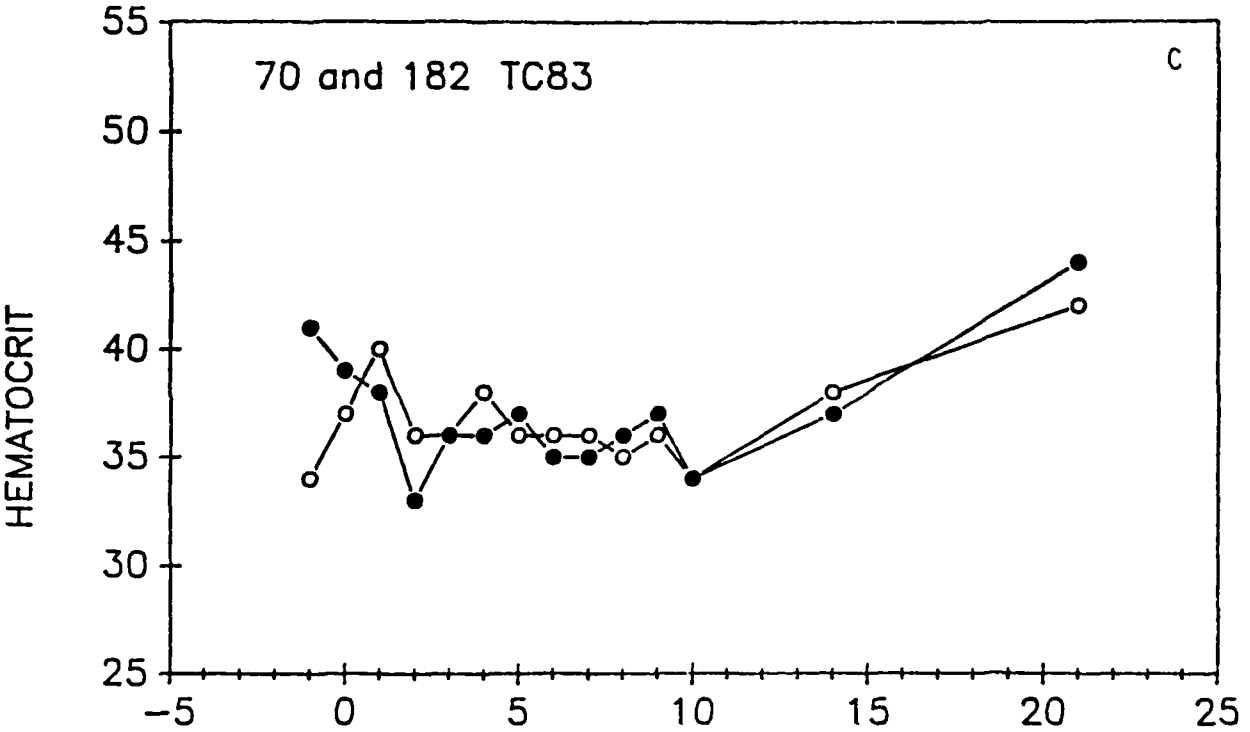
15
20
25

Fig. 7


70 and 182 TC83
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5
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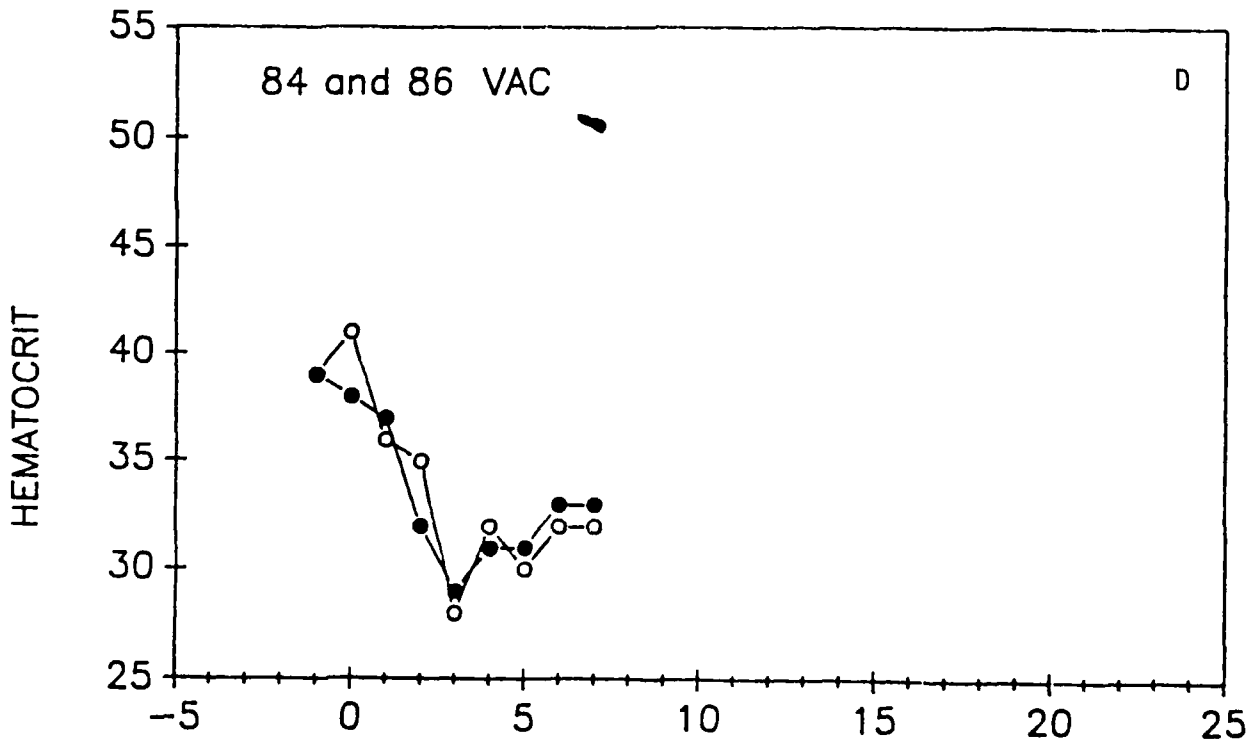
15
20
25


84 and 86 VAC
D
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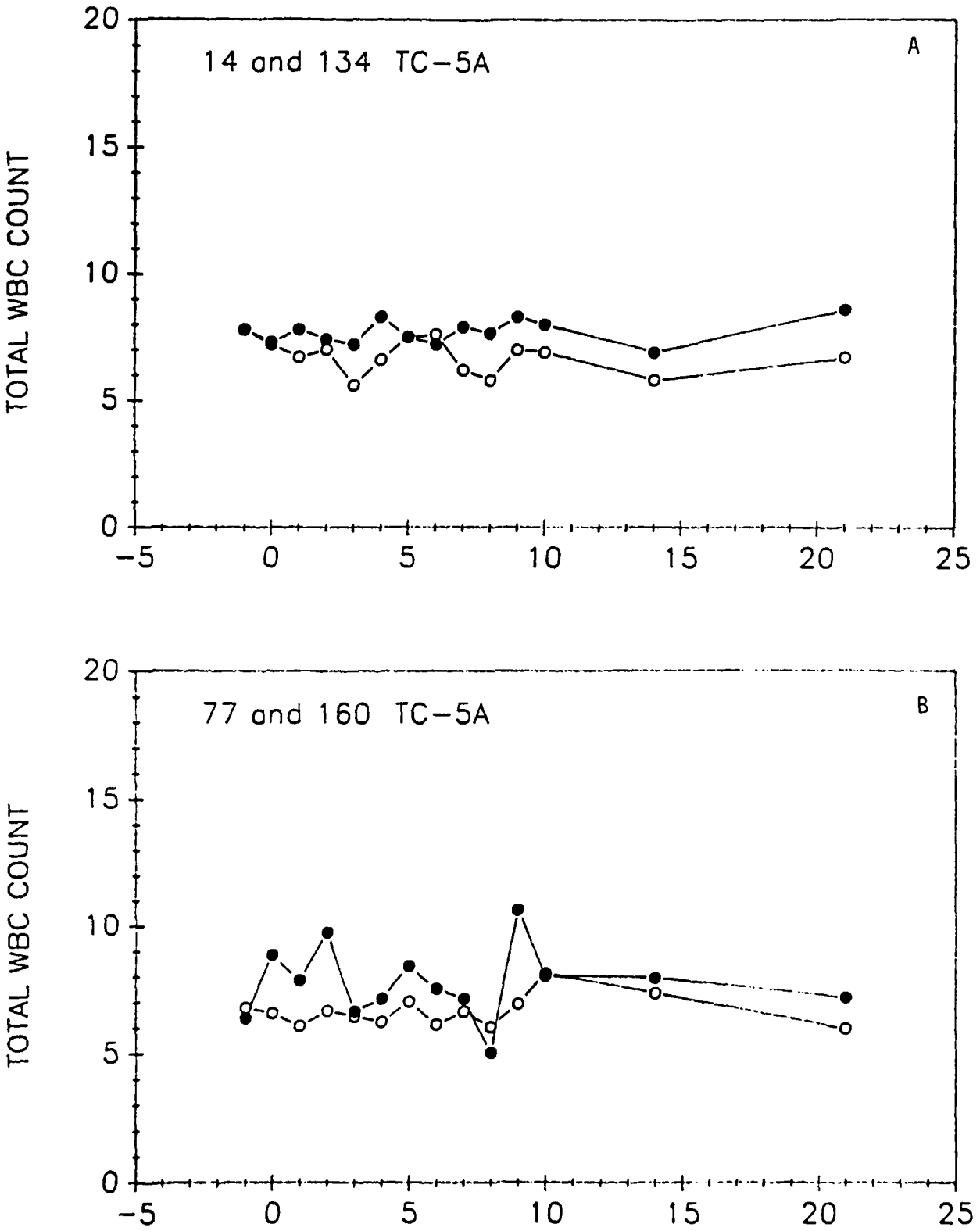
15
20
25

Fig. 8


14 and 134 TC-5A
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TOTAL WBC COUNT
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15
10
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-5
0
5
10
15
20
25
77 and 160 TC-5A
B
TOTAL WBC COUNT
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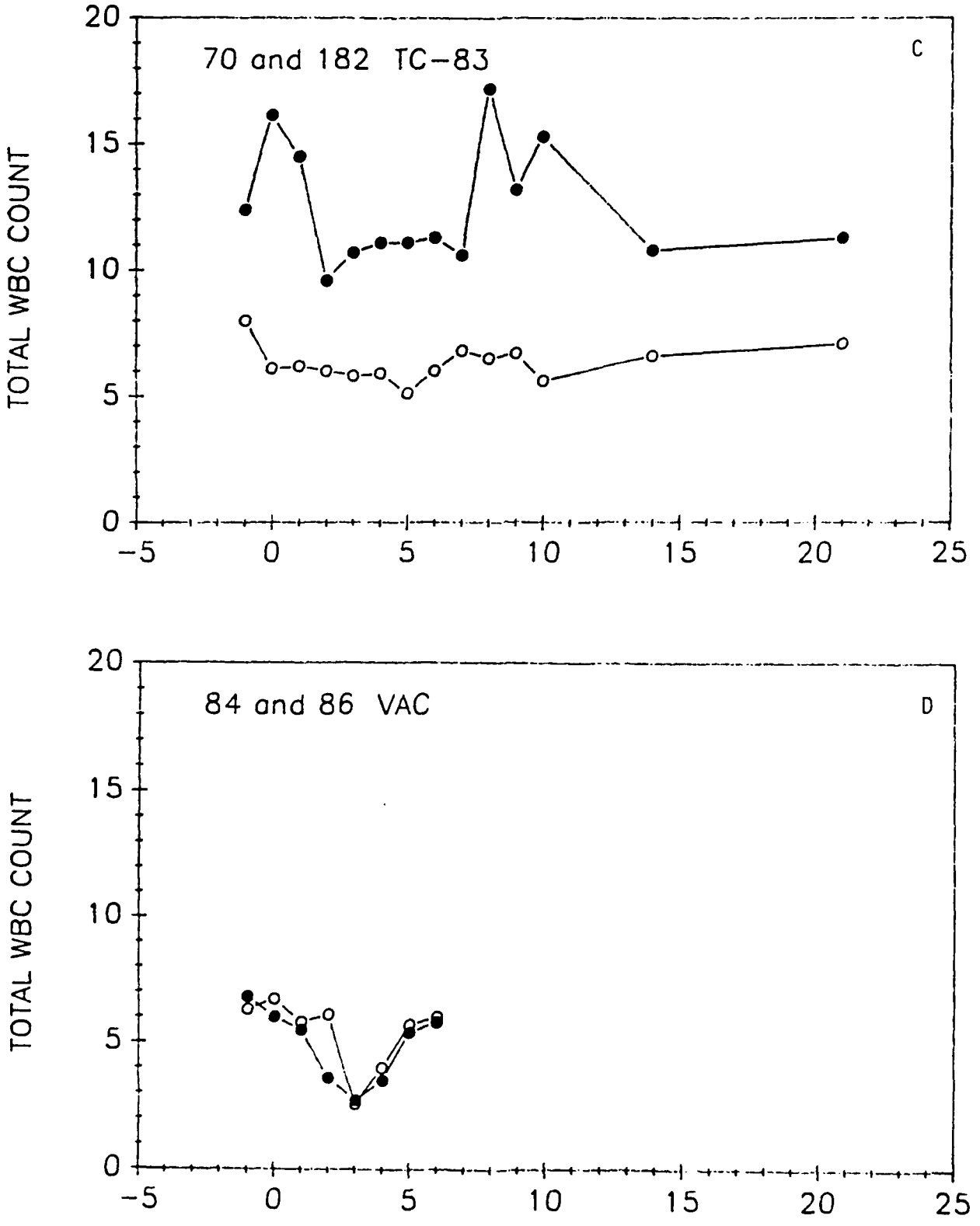
15
10
5
0
-5
0
5
10
15
20
25

Fig. 8


70 and 182 TC-83
C
TOTAL WBC COUNT
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84 and 86 VAC
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TOTAL WBC COUNT
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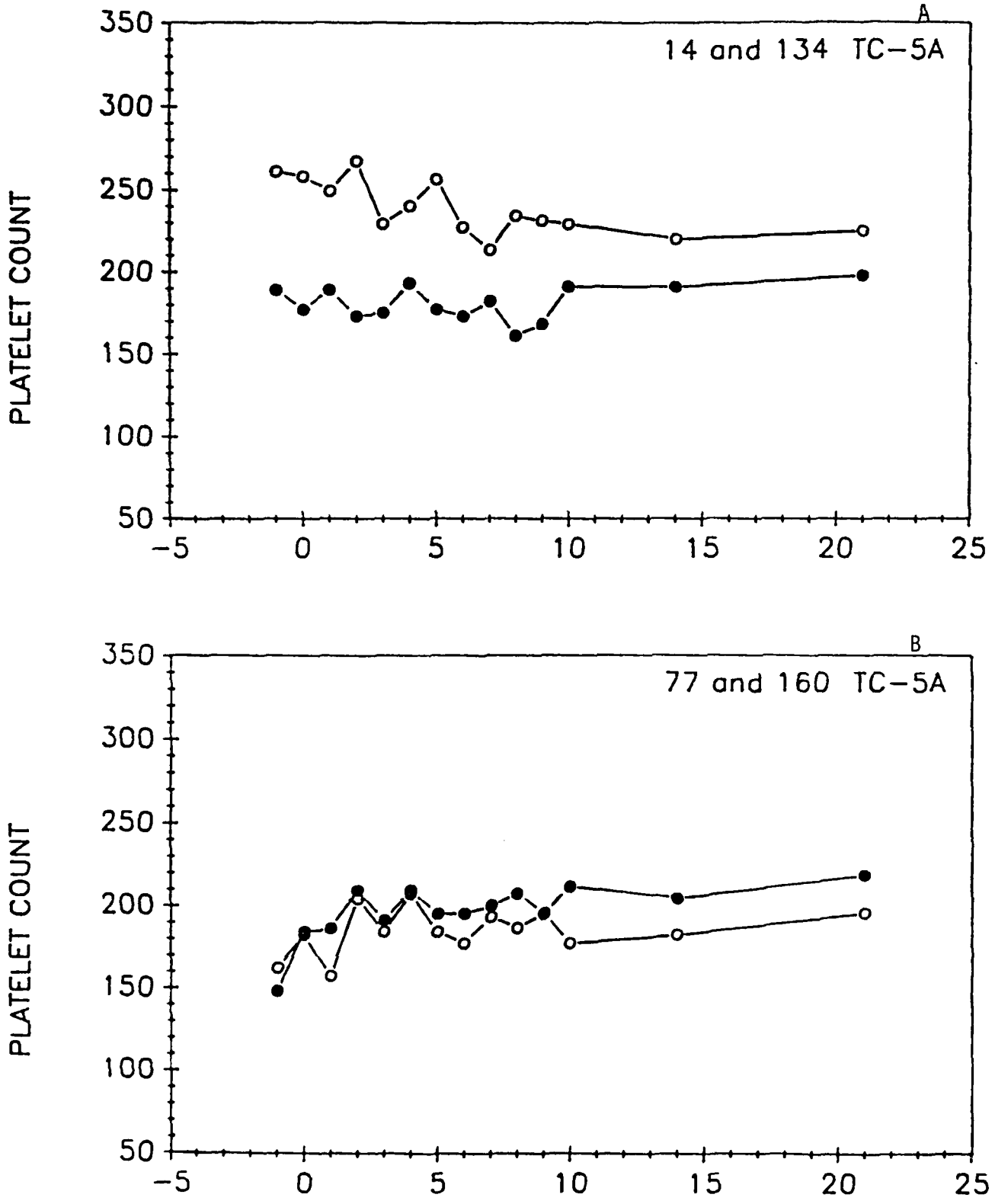
20
25

Fig. 9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200
150
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0
5
10
15
20
25
B
77 and 160 TC-5A
PLATELET COUNT
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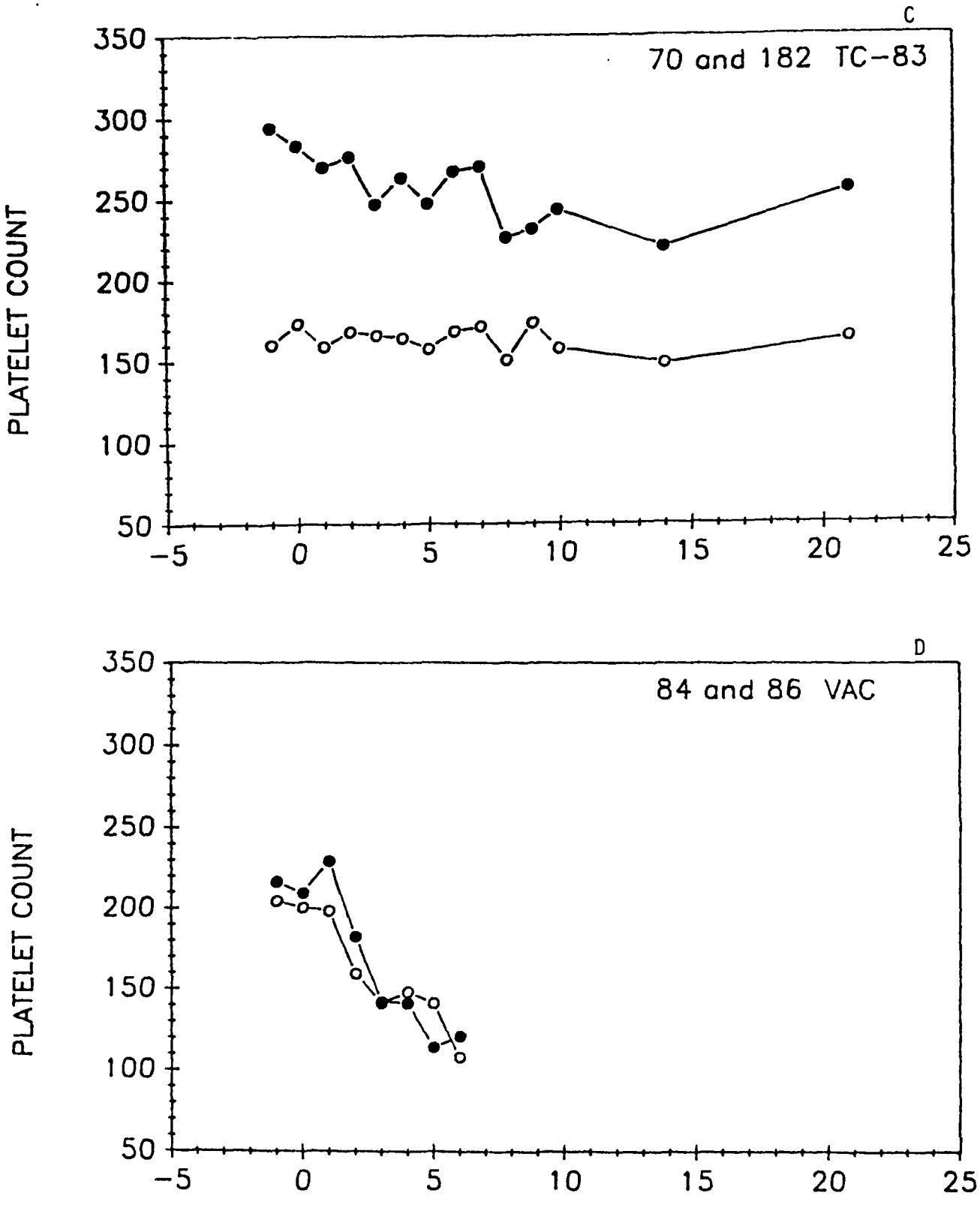
150
100
50
-5
0
5
10
15
20
25

Fig. 9


C
70 and 182 TC-83
PLATELET COUNT
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300
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200
150
100
50
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5
10
15
20
25
D
84 and 86 VAC
PLATELET COUNT
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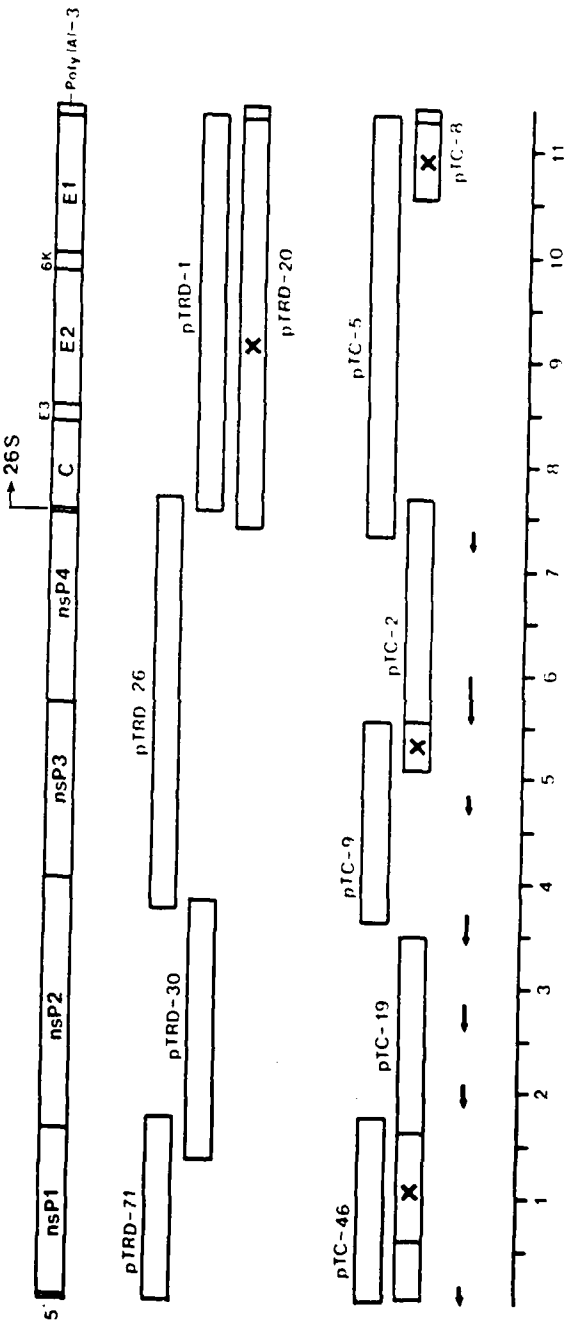
300
250
200
150
100
50
-5
0
5
10
15
20
25

Fig. 10


5'
nsP1
nsP2
nsP3
nsP4
C
26S
E3
E2
6K
E1
Poly(A)-3
pTRD-71
pTRD-30
pTRD 26
pTRD-1
pTRD-20
pTC-46
pTC-19
pTC-9
pTC-2
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2
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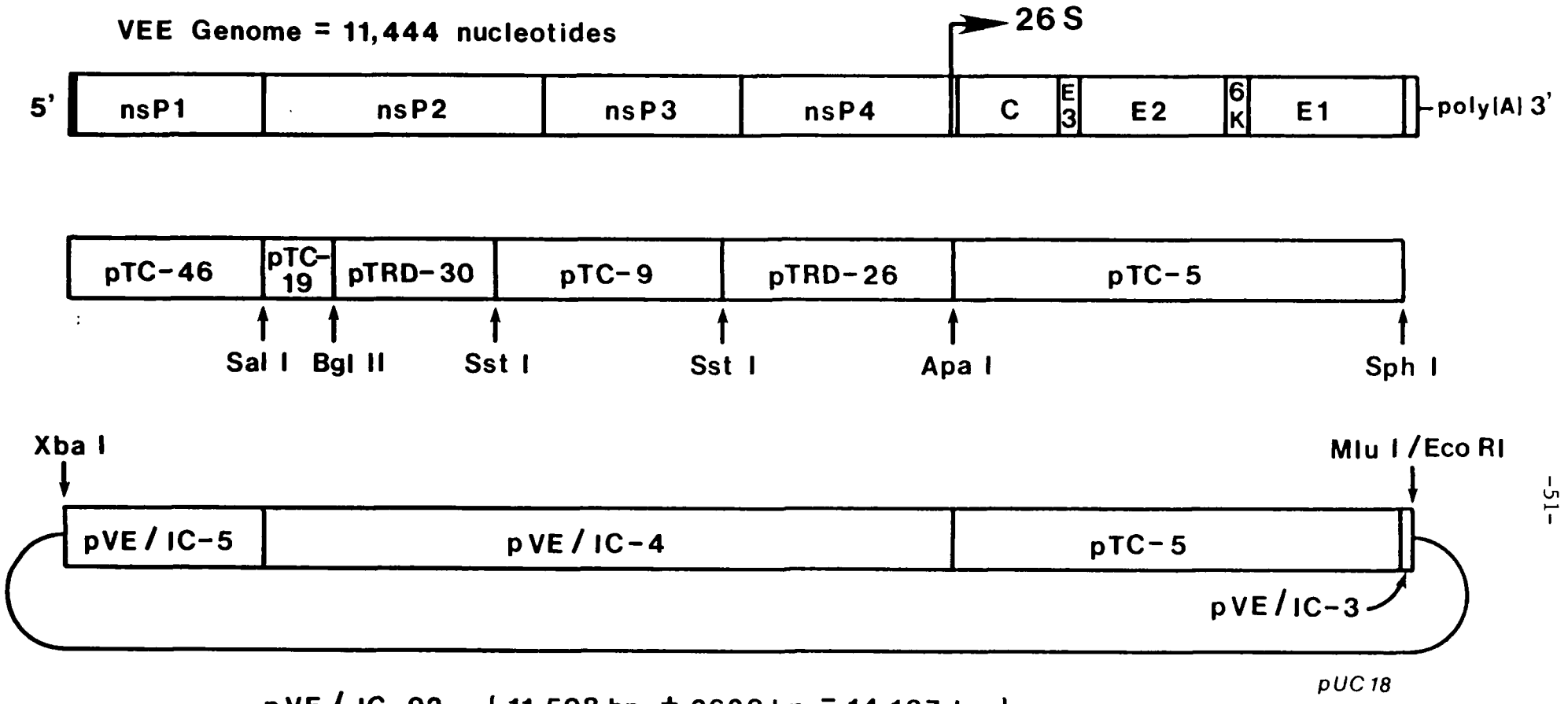
9
10
11

Fig. 11



pVE / IC-92 [11,508 bp + 2629 bp = 14,137 bp]

Fig. 12

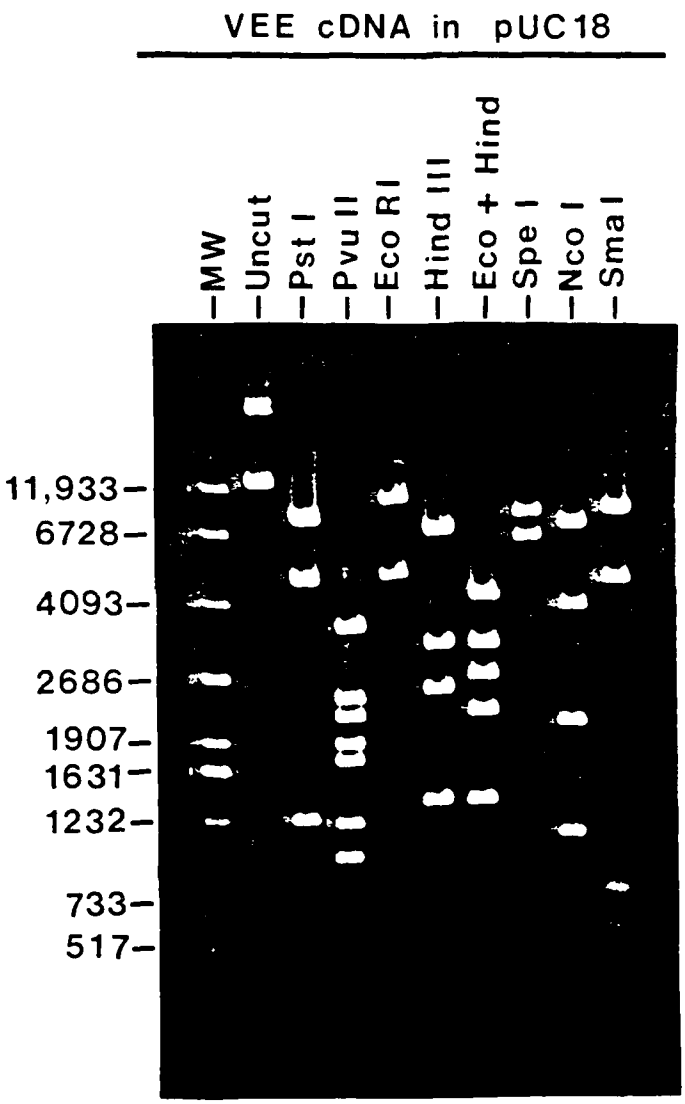

VEE cDNA in pUC18
MW
Uncut
Pst I
Pvu II
Eco RI
Hind III
Eco + Hind
Spe I
Nco I
Sma I
11,933
6728
4093
2686
1907
1631
1232
733
517

Fig. 13

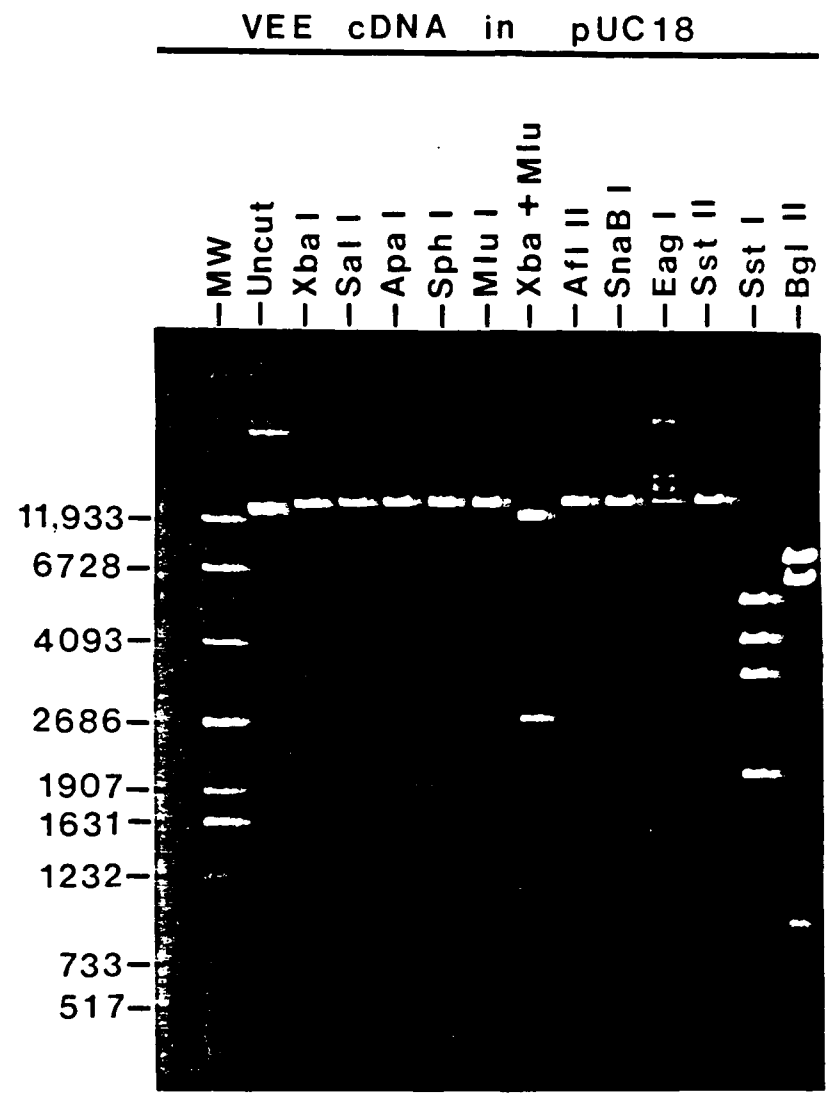

VEE cDNA in pUC18
MW
Uncut
Xba I
Sal I
Apa I
Sph I
Mlu I
Xba + Mlu
Afl II
SnaB I
Eag I
Sst II
Sst I
Bgl II
11,933
6728
4093
2686
1907
1631
1232
733
517

Fig. 14